ERDC/TN SAV-06-1
January 2006

Applications and Limitations of Micropropagation for the Production of Underwater Grasses

by Steve Ailstock and Deborah Shafer

DEFINITION: Micropropagation is a technique that manipulates small quantities of axenic plant material, ranging from single cells to stem segments, under conditions favorable to the formation of new plants. It has proven to be the most efficient and cost-effective method of propagating large numbers of clonal offspring for many agronomic crops, including both herbaceous and woody perennial species. Older and simpler techniques of cloning plants (cuttings, grafting, and division of parent stock material) are limited by seasonal constraints and the natural formation of new plant structures. Micropropagation, on the other hand, allows the year-round production of new plants at rates significantly higher than that achievable by all other methods. The plants produced are genetically uniform, vigorous, and free from associations with other organisms, an attribute particularly useful for the culture of underwater grasses where contaminating organisms can dominate other types of production systems.

PURPOSE: This technical note outlines the applications and limitations of micropropagation for the production of submersed aquatic vascular plants used for habitat restoration, experimentation, and educational demonstrations of the importance of these plant communities. Submersed aquatic vascular plants are unique members of the plant kingdom because they are secondarily adapted to life underwater (Raven 1984, Den Hartog 1970). The compliment of adaptations, both anatomical and physiological, that allows terrestrial plants to balance the need to conserve water against the need to obtain inorganic carbon from the atmosphere was driven by the distinct differences between the aquatic and gaseous environments (Ailstock 1996, Kramer and Boyer 1995, Johnson et al. 1991). Those flowering vascular plants that returned exclusively to underwater environments successfully adapted this terrestrial architecture to the aquatic environment. However, this evolution occurred well in advance of human influences on water quality. Many of the applications of micropropagation to submersed aquatic angiosperms or "underwater grasses" are a result of the need to manipulate these plants under conditions that are unique and often less than optimal.

A prerequisite for a thorough understanding of any organism is the availability of stock that is free of perturbation. Few aquatic environments exist today where water quality is sufficient to support the maximum growth of these plants (Sheridan et al. 1998, Anonymous 1992). Excess runoff from the land, nutrient enrichment, and contaminants stress the plants directly and modify the environment in ways that favor competitive autotrophs and herbivores (Kemp et al. 1983, Stevenson and Confer 1978). In many systems, the consequences of these changes have been major declines of underwater grass communities and exceptional stress on those that persist (Orth and Moore 1984). The plants in many of these systems are small, sparse, exhibit characteristics of stress morphology, and are heavily contaminated with a variety of microorganisms. Even in the best of environments, the aquatic environment poses unique challenges for vascular plants that have substantially slower growth rates than competitive autotrophs and epiphytes. The delicate balance of life in these systems complicates plant culture under artificial systems where bursts of excessive growth of microorganisms can overwhelm the normal growth of underwater plants. The value of having plant sources free of these contaminants cannot be under-estimated. Thus, the principal application of micropropagation is simply the year-round availability of large numbers of healthy plants that

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JAN 2006	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Applications and Limitations of Micropropagation for the Production of Underwater Grasses	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Army Engineer Research and Development Center Vicksburg, MS**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**SAR**	**11**	

can be used for study under tightly controlled conditions. Once the protocols for micropropagation systems are defined, the abundance of plants produced can be used in various types of demonstration and restoration projects (Deleon et al. 1995, Bird et al. 1994, Koch and Durako 1989); bioassays (Hall et al. 1996, Durako et al. 1995); and scientific study (Nandi et al. 1999, Ailstock et al. 1991, Wetzel and McGregor 1968).

EQUIPMENT AND PROCEDURES: The development of micropropagation systems requires resources common to most labs dedicated to microbiology. These resources include those used for the preparation of sterile media including balances, pH meters, autoclaves, filtration systems for cold sterilization of media, and environmental chambers suitable for media storage. Laminar flow hoods are needed to provide a sterile work environment for explant production and repeated subdivisions of stock during the propagation phase of plant production. Growth chambers capable of providing photosynthetically active radiation (PAR) under various photoperiods and maintaining a range of temperature regimes are also necessary. The rigor of these environmental controls depends upon the anticipated application of the plants produced. Finally, for those applications that involve production for field establishment, a variety of systems ranging from buckets and shallow pans to flow-through aquaculture tank systems are needed to grow plants to the sizes needed for transplantation.

The procedural requirements for developing micropropagation systems are well established (see additional references in the "Sources of Additional Information" section.) The first requirement is to select either a species or ecotype that possesses the attributes desired by the propagator. For agricultural applications, these characteristics often include economic attributes such as high yield varieties, disease resistance, drought tolerance or preservation of a desirable mutation (Withers and Alderson 1986, Zimmerman 1986). Ecological applications like those concerned with underwater grasses are often as simple as the selection of a keystone species for a particular type of habitat based on distribution and tolerance of fluctuating salinities (Sheridan et al. 1998). Next, the life cycle of the chosen species is evaluated to identify plant tissues that are somewhat protected and free of abundant living contaminants like bacteria and fungi. Preferred tissues may include meristematic cells, the apical buds of photosynthetic stem and rhizomes, the meristems of over-wintering structures, or embryonic tissue that is protected by an intact seed coat (Cassells 1997, Kyte and Kleyn 1996, Madsen 1985).

Once selected, these tissues, called explants, are exposed to combinations of surface sterilants, antibiotics, and fungicides to identify combinations that kill associated living contaminants without killing the plant tissues selected for manipulation (Cassells 1997). When a reliable source of axenic material is obtained, the time-consuming process of identifying a propagation medium that will support reproductive growth begins. In general, various media differing in concentrations of mineral nutrients, carbohydrates, plant growth regulators, pH, salinity, and incubation temperature are inoculated with explants until a positive reproductive growth response is obtained (Rosen 2000; Bird et al. 1991, 1993, 1996; Bird and Jewett-Smith 1994; Koch and Durako 1991; Durako and Moffler 1984; Moffler and Durako 1984). Then, the media is refined in order to develop a recipe for achieving multiple reproductive structures at acceptable rates of growth.

Once a propagation medium has been developed, plants are produced by subdividing the axenic plants for culture on a growth medium. Growth media lack the carbohydrate sources and plant growth regulators of the propagation medium but retain concentrations of the essential elements required by all plants. The pH and salinity of this medium is that preferred by the species in its natural environment (Woodhead and Bird 1998, Smith 1993, Thursby 1984, Wetzel and McGregor 1968). A defined growth medium allows production of new plants for a particular application at an exponential rate free of all seasonal constraints (Woodhead and Bird 1998). For experiments, plants may be used directly in the growth medium or transferred to mesocosms for bioassays. Those used for demonstration and restoration projects make be taken directly from the growth medium for plants or used to develop transplants in the various containers used

for field establishment. The process used to produce sago pondweed (*Stuckenia pectinatus; syn. Potamogeton pectinatus)* is summarized in Figures 1-6.

Figure 1. Stock selected for micropropagation should exhibit desirable qualities such as stress resistance, vigor, environmental persistence, or other traits that impart plant hardiness under ambient field conditions

Figure 2. Turions of sago pondweed have protected meristems that are prime sources of initial material for disinfestations of living contaminants prior to culture

Figure 3. Removal of living contaminants like bacteria and fungi is achieved by exposure to surface sterilants, antibiotics, and fungicides in a sterile working environment under a laminar flow hood

Figure 4. Once a positive growth response is obtained, the process of media refinement is facilitated

Figure 5. The process of subculturing new plants from axenic cultures can occur in a variety of culture vessels

Figure 6. Production from micropropagation systems is exponential and can be adjusted to produce plants in virtually unlimited quantities

APPLICABILITY

Restoration and mitigation projects. One application of micropropagation of submersed aquatic plants is to provide planting stock for field establishment in restoration or mitigation projects. Micropropagation allows production of large numbers of aquatic plants as pure cultures without seasonal constraints. Use of cultured planting stock would reduce impacts to donor beds caused by harvest of wild stocks and ensure availability of plants to meet sporadic project schedules (Fonseca et al. 1998).

Plants may be harvested directly from the growth medium or they can be used to grow larger transplants in a variety of containers in greenhouse or flow-through culture systems. Regardless of the type of preparation for field establishment, consideration must be given to the logistics of transport, since these plants are prone to rapid desiccation and damage as they lack both water-conserving adaptations and abundant mechanical tissue (see Figures 7-13).

Dose/response experiments. In the laboratory environment, dose/response experiments where the plants are exposed to serial differences in such things as essential nutrients, pH, salinity, temperature, photoperiod, light intensity, and inorganic carbon source define the basic requirements that allow these plants to reach their genetic potential. The results of these experiments are directly applicable to field conditions as a predictor of potential plant performance. Once basic growth requirements are established, multivariant experiments can define the synergistic effects of different environmental parameters or contaminants. When plants are exposed to serial dilutions of herbicides, concentrations that limit growth and kill the

plants can be expressed as the effective concentration or the lethal concentration in units of plant biomass. Taken collectively, these types of experiments have been shown to accurately identify the effects of ambient environmental conditions on plant growth because plant responses do not need to be interpreted based on the influences of other organisms that might be present.

Figure 7. Plants produced in micropropagation systems can be harvested directly for use in field restoration projects

Figure 8. Plants can be transported to greenhouse aquaculture systems for further growth

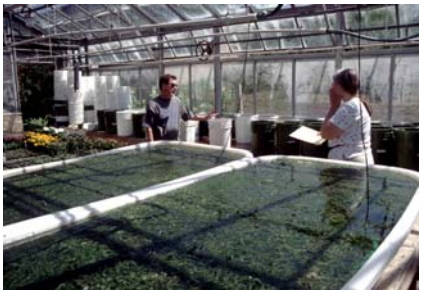

Figure 9. Greenhouse aquaculture system for production of containerized transplants

Figure 10. Submersed aquatic angiosperms lack mechanical and water conservation tissues, which makes them vulnerable to physical damage and desiccation. Insulated coolers are a convenient method of protecting plants during their transport to field locations

Figure 11. Plants can be taken directly from micropropagation culture systems as bare root transplants

Figure 12. Containerized transplants are separated and prepared for planting

Figure 13. Field restorations in the mid-Chesapeake Bay are frequently conducted on fine sediments in 0.25-1.0 m of water. Disturbance of sediments coupled with natural turbidity in these environments limits visibility. Large numbers of volunteers are often used to compensate for the loss of efficiency and to reduce restoration costs

The interpretation of plant dose/response experiments can be challenging, however (Hall et al 1996, Johnson and Bird 1995). The most common endpoints in dose response experiments involving animals are mortality, biomass, consumption, and morphology (American Society for Testing and Materials (ASTM) 1997, Siesko et al. 1997). These are not necessarily convenient or useful endpoints for assessing

plant responses. Unlike animals, where growth is distributed over the entire organism, cell division and growth of herbaceous perennial plants are isolated in tissues called meristems. Throughout the growing season, these meristems produce new cells that enlarge and differentiate into roots, rhizomes, photosynthetic shoots, and over-wintering structures. As new plant structures are produced, older parts senesce and die. The sequence and rate of death of these parts are determined by the particular structure, age of the structure, and the degree of plant stress. Leaves and roots usually experience higher rates of turnover than rhizomes and over-wintering structures, which tend to be more persistent. Therefore, the loss of parts, their replacement, and subsequent entrance into dormancy are natural survival features of perennial plants that are not found in animals. For example, when stressed, plant tissues in the water column will often die while those in the sediment will survive, albeit in a dormant condition. Thus, stressed plants may exhibit low mortality (parts needed for resumption of growth remain alive), little change in biomass (comprised of mostly dead tissue), and the morphology may be similar to periods of naturally induced dormancy. Thus, endpoints to identify the consequences of a stress on perennial plants must be chosen in light of a pre-selected standard. In this example, if survival is an acceptable consequence of stress, then low mortality makes the stressor acceptable at test levels. If, however, continuous active growth is the standard of acceptance, then the effects of the stressor would be viewed as unacceptable. This would require measurement of a physiological endpoint such as oxygen evolution, a trait associated with active photosynthetic growth, to estimate the degree of the effect (see Figures 14 and 15).

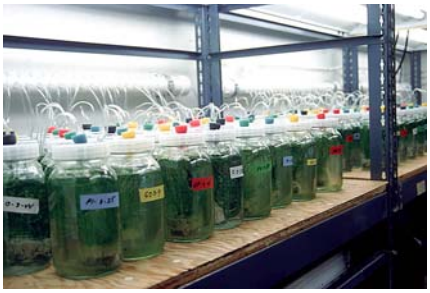

Figure 14. Axenic plant cultures are highly controlled systems that can be exploited for measuring the effects of parameters, like herbicide concentration, on plant growth

Figure 15. Endpoints to evaluate the effects of physical and chemical environmental factors through dose/response experiments must be selected to accommodate the metabolism of the plant being evaluated

Bioassay systems. Micropropagation can provide large numbers of healthy, undamaged plants to assess ecosystem health. These plants can be placed in natural environments to determine whether or not the ambient conditions will allow the plants to survive, or how well they grow relative to other sites or conditions. Alternatively, sediments and water can be collected from natural environments and used in the lab to incubate plants and assess growth. However, caution must be exercised to avoid using their growth responses to make generalizations about environmental quality, especially with respect to sediment-borne contaminants and toxic compounds. The characteristics of sediments that favor the accumulation of many chemicals are also those that favor the accumulation of essential plant nutrients. Thus, suppression of growth by contaminants could be masked due to the stimulus provided by high concentrations of nutrients.

Demonstration and education. Micropropagation techniques that allow year-round production of plants make these systems attractive for demonstration and education events, which often do not coincide with plant availability under field conditions. Such activities have proven to be important for developing the grass roots support needed to preserve and restore underwater grass communities. Several states now make underwater grass production a part of their formal education and community outreach programs. Those that have relied exclusively on other types of culture systems or sources of plants have experienced problems both with plant availability and the production of plants at times appropriate for use. In contrast,

micropropagation systems can provide a reliable year-round source of plants in any number necessary to satisfy program objectives. Moreover, the plants produced via micropropagation generally respond better and persist longer in the closed systems employed for these demonstration/education initiatives because of their larger size and lack of damage (see Figures 16 and 17).

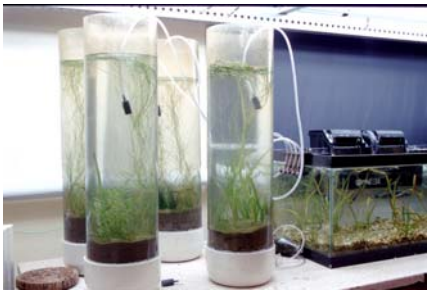

Figure 16. Plants produced in micropropagation systems are free of the seasonal constraints of field collections and are available year round

Figure 17. *Stuckenia pectinatus* plants are grown in buckets to produce turions that can be used in field restoration projects

IMPORTANT CONSIDERATIONS FOR PLANNING: Since submerged aquatic plants are important components of many shallow-water aquatic ecosystems, restoration of these plant communities has been a major focus in many areas, particularly in the Chesapeake Bay. In some cases, restoration projects have been limited by a lack of available planting stock, and concerns regarding potential impacts to donor beds of wild stock. Micropropagation systems offer a number of advantages, including the cost-effective production of virtually unlimited numbers of healthy plants without the seasonal constraints associated with other sources of plant material. However, there are also some limitations that should be considered when making decisions regarding sources of plant stock.

Cost-effectiveness. Compared with other methods used for the propagation of herbaceous perennial plants, micropropagation systems are second only to seeds for the cost-effective production of large numbers of new plants. Table 1 presents estimated time requirements for production of submersed aquatic plants using the conventional approaches of field harvesting native stock, vegetative propagation by cuttings or divisions, micropropagation, and seeds. At a minimum, micropropagation can increase production by 100 percent compared with all other cloning methods. Although not as efficient as seed production, the axenic plants produced by micropropagation offer a variety of advantages over all other methods including seed.

Table 1
Time Requirements for Production of Submersed Aquatic Plants Used in Restoration Projects

Production Elements	Harvested Plants	Vegetative Propagation	Micropropagation Facilities	Seeds
Harvest	8-10 min	2-5 min	0.2-1 min	0.1-5 sec
Propagation	1-2 min	1-2 min	1-2 min	NA
Prepare planting unit	1-2 min	1-2 min	1-2 min	0.1-3 sec
Delivery/planting	2-5 min	2-5 min	1-3 min	0.1-3 sec
Total	**12-19 min**	**6-14 min**	**3.2-8 min**	**0.3-11 sec**

Genetic selection and diversity. Like all forms of vegetative propagation, these systems produce genetically uniform clones. While advantageous in many applications, clones do not exhibit the range of genetic variability present in a species (Durako and Moffler 1981). Thus, conclusions regarding tolerances to environmental perturbations must be drawn in light of this limitation unless a number of ecotypes are cloned for use in experimentation. Even then the argument can be made that other ecotypes could respond differently under the same sets of conditions. Therefore, micropropagation systems should strive to include as many different genotypes as possible as initial sources of plant material in order to increase genetic diversity.

Micropropagation systems also present an opportunity for the development of "genetically improved" strains, which are resistant to stress or disease. However, the introduction of altered genotypes to natural populations remains a controversial issue in population ecology. While selection of plant strains with specific traits is common in the agricultural community, application of such practices to the management of wild plant populations is untested. Such an undertaking would require not only a thorough understanding of the existing population genetic structure and those site-specific factors influencing it, but also a definitive basis for assessing what constitutes 'improvement' (Fonseca et al. 1998).

Target species availability. Despite considerable effort, micropropagation systems have not been successfully developed for all species of interest. In general, species adapted to fresh and brackish water environments have been more successful. Seagrasses, defined as those underwater grasses that are found in highly saline marine waters, have proven especially difficult to culture. Some of the difficulties must be attributed to the problems of osmotic regulation associated with the use of detached plant parts (McMillan 1996, Koch and Durako 1989). There remains a serious need to understand the basic biology and reproductive physiology of these unique organisms. Yet, once these systems are in place, the potential returns more than justify continued efforts to bring other species of submersed aquatic plants into production by these methods.

POINTS OF CONTACT: For more information, contact Dr. Steve Ailstock, Anne Arundel Community College, MD (*smailstock@aacc.edu*), or Ms. Deborah Shafer, U.S. Army Engineer Research and Development Center, Vicksburg, MS (601-634-3650, *Deborah.J.Shafer@erdc.usace.army.mil*).

REFERENCES:

Ailstock, M. S. (1996). "Classification, culture, and compartmentation strategies of submersed aquatic plants," Ph.D. diss., University of Maryland, College Park, MD.

Ailstock, M. S., Fleming, W. J., and Cooke, T. J. (1991). "The characterization of axenic culture systems suitable for plant propagation and experimental studies of the submersed aquatic angiosperm *Potamogeton pectinatus* (sago pondweed)," *Estuaries* 14(1), 57-64.

American Society for Testing and Materials. (1997). "Standard Guide for Conducting Static, Axenic, 14 Day Phytotoxicity Tests in Test Tubes with the Submersed Aquatic Macrophyte, *Myriophyllum sibiricum* Komarov," *ASTM Standards*, American Soc. Testing and Materials, PA. Vol. 11.05, E-1913.

Anonymous. (1992). "Chesapeake Bay submersed aquatic vegetation habitat requirements and restoration targets: A technical synthesis," Report CBP/TRS 83/92/ Printed by EPA for Chesapeake Bay Program, Annapolis, MD.

Bird, K. T., and Jewett-Smith, J. (1994). "Development of a medium and culture system for in vitro propagation of the seagrass *Halophila engelmanni*," *Canadian Journal of Botany* 72(10), 1503-1510.

Bird, K. T., Brown, M. S., Henderson, T. T., O'Hara, C. E., and Robbie, J. M. (1996). "Culture studies of *Ruppia maritima* L. in bicarbonate and sucrose based media," *Journal of Experimental Marine Biology and Ecology* 199(2), 153-164.

Bird, K. T., Cody, B., and Jewett-Smith, J. (1991). "Effects of salinity on micropropagation of *Ruppia maritima*." *11th Annual Estuarine Research Conference, San Francisco, November 10-14, 1991*, 67.

Bird, K. T., Cody, B., Jewett-Smith, J., and Kane, M. E. (1993). "Salinity effects on *Ruppia maritima* L. cultured in vitro." *Botanica Marina* 36(1), 23-28.

Bird, K. T., Jewett-Smith, J., and Fonseca, M. S. (1994). "Use of in vitro propagated *Ruppia maritima* for seagrass meadow restoration," *Journal of Coastal Research* 10(3), 732-737.

Cassells, A. C. (1997). "Pathogen and microbial contamination management in micropropagation," *Development in plant pathology* (12), Kluwer Academic Publishers. Boston, MA.

Deleon, M. F., Durako, M. J., Shup, J. J., and Daeschner, S. (1995). "Real-world considerations for micropropagating *Ruppia maritima* L. (widgeon grass) for seagrass restoration projects." *Proc. 22nd Ann. Conf. Ecosystem Restoration Creation*, Hillsborough Community College, Inst. Florida Studies, Plant City, FL, 27-38.

Den Hartog, C. (1970). *The sea-grasses of the world.* North-Holland Publishing Company, Amsterdam.

Durako, M. J., and Moffler, M. D. (1981). "Variation in *Thalassia testudinum* seedling growth related to geographic origin," *Proceedings of the 8th Annual Conference on Wetlands Restoration and Creation*. R. H. Stovall, ed., Hillsborough Community College, Tampa, FL.

Durako, M. J., and Moffler, M. D. (1984). "Qualitative assessment of five artificial growth media on growth and survival of *Thalassia testudinum* (Hydrocharitaceae) seedlings." *Proc. 11th Ann. Conf. Wetland Restoration and Creation*. F. J. Webb, ed., 17-18 May 1984, Hillsborough Comm. Coll., Tampa, FL, 73-92.

Durako, M. J., Shup, J. J., Deleon, M. F., and Daeschner, S. (1995). "A bioassay approach to seagrass restoration." *Proc. 22nd Ann. Conf. Ecosystem Restoration Creation*. Hillsborough Community College, Inst. Florida Studies, Plant City, FL., 44-56.

Fonseca, M. S., Kenworthy, J., and Thayer, G. (1998). "Guidelines for the conservation and restoration of seagrasses in the United States and adjacent waters," NOAA Coastal Program Decision Analysis Series No. 12. NOAA Coastal Ocean Office, Silver Spring, MD.

Hall, L. W., Jr., Ailstock, M. S., and Anderson, R. D. (1996). "Standard operating procedures for conducting sub-chronic aquatic toxicity tests with sago pondweed *Potamogeton pectinatus*: A submersed aquatic angiosperm," Final report, University of Maryland, Maryland Agricultural Experimental Station, Wye Research and Education Center, Queenstown, MD.

Johnson, J. R., and Bird, I. T. (1995). "The effects of the herbicide atrazine on *Ruppia maritima* growing in autotrophic versus heterotrophic cultures," *Botanica Marina* 38, 307-312.

Johnson, J. R., Melkonian, J. J., Thornley, J. H. M., and Riha, S .J. (1991). "A model of water flow through plants incorporating shoot/root "message" control of stomatal conductance," *Plant, Cell Environ*. 14, 531-544.

Kemp, W. M., Boynton, W. R., Twilley, R. R., Stevenson, J. C., and Means, J C. (1983). "The decline of submerged vascular plant in the upper Chesapeake Bay: Summary of results concerning possible causes," *Marine Technical Society Journal* 17, 78-89.

Koch, E. W., and Durako, M. J. (1989). "Seagrass micropropagation: A step closer to restoration with "in vitro" plants (Florida)," *Restoration and Management* 7(2), 99-100.

Koch, E. W., and Durako, M. J. (1991). "In vitro studies of the submersed angiosperm *Ruppia maritima*: Auxin and cytokinin effects on pant growth and development," *Marine Biology* 110, 1-6.

Kramer, P. J., and Boyer, J. S. (1995). *Water relations of plants and soils*. Academy Press, Inc., San Diego, CA.

Kyte, L., and Kleyn, J. G. (1996). *Plants from test tubes: An introduction to micropropagation*. 3rd ed., Timber Press, Portland, OR.

Madsen, J. D. (1985). "The use of vegetative propagules for sterile cultures of aquatic macrophytes," *The Michigan Botanist* 24, 169-173.

McMillan, C. (1996). "Seagrasses in experimental culture." *Seagrass Biology: Proc. International Workshop*. J. Kuo, R. C. Phillips, D. I. Walker, and H. Kirkman, ed., Rottnest Island, Univ. of Western Australia, 25-29 January 1996, 11-12.

Moffler, M. D., and Durako, M. J. (1984). "Axenic culture of *Thalassia testudinum* Banks ex Konig (Hydrocharitaceae)," *American Journal of Botany* 71 (10), 1455-1460.

Nandi, S. K., Kumar, A., and Plani, L. M. S. (1999). "Role of plant tissue culture in biodiversity conservation and economic development," Himavikas occasional publication; No. 15. Nainital: Gyanodaya Prakashan for the G.B. Plant Institute of Himalayan Environment & Development.

Orth, R. J., and Moore, K. A. (1984). "Distribution and abundance of submerged aquatic vegetation in Chesapeake Bay: An historic perspective," *Estuaries* 7, 531-540.

Raven, J. A. (1984). "Energetics and transport in aquatic plants," MBL Lectures in Biology Volume 4. Alan R. Liss, Inc., New York.

Rosen, D .J. (2000). "Culture media affects the growth of nursery-grown shoalgrass (Texas)," *Ecological Restoration* 18(2), 202-203.

Sheridan, P., McMahan, G., Hammerstrom, K., and Pulich, W. (1998). "Factors affecting restoration of *Halodule wrightii* to Galveston Bay, Texas," *Restoration Ecology* 6(2), 144-158.

Siesko, M. M., Fleming, W. J., and Grossfeld, R. M. (1997). "Stress protein synthesis and peroxidase activity in a submersed aquatic macrophyte exposed to cadmium," *Environmental Toxicity and Chemistry* 16(8), 1755-1760.

Smith, C. S. (1993). "A bicarbonate-containing medium for the solution culture of submersed plants," *Canadian Journal of Botany* 71 (12), 1584-1588.

Stevenson, J. C., and Confer, N. M. (1978). "Summary of available information on Chesapeake Bay submerged vegetation," U.S. Fish and Game Biological Services Program. FWS/OBS 78/66.

Thursby, G. B. (1984). "Nutritional requirements of the submerged angiosperm *Ruppia maritima* in algae-free culture," *Marine Ecology Progress Series* 16 (1/2), 45-50.

Wetzel, R. G., and McGregor, D. L. (1968). "Axenic culture and nutritional studies of aquatic macrophytes," *Am. Midland Naturalist* 80(1), 52-64.

Withers, L. A., and Alderson, P. G. (1986). *Plant tissue culture and its agricultural applications*. Butterworths, Boston, MA.

Woodhead, J. L., and Bird, K. T. (1998). "Efficient rooting and acclimation of micropropagated *Ruppia maritima* Loisel," *Journal of Marine Biotechnology* 6, 152-156.

Zimmerman, R. H. (1986). "Tissue culture as a plant production system for horticultural crops." *Current plant science and biotechnology in agriculture*. Kluwer Academic Publishers. Boston, MA.

BIBLIOGRAPHY:

Bajaj, Y. P. S. (1997). "High-tech and micropropagation." *VI. Biotechnology in agriculture and forestry*. Springer, New York.

Conger, B. V. (1981). *Cloning agricultural plants via in vitro techniques*. CRC Press, Boca Raton, FL.

Dodds, J. H., and Roberts, L. W. (1995). *Experiments in plant tissue culture*. 3rd ed. Cambridge University Press, Cambridge, England.

Gamborg, O. L., and Wetter, L. R. (1975*). Plant tissue culture methods*. National Research Council of Canada, Saskatoon, Sask.

Kyte, L. (19870. *Plants from test tubes: An introduction to micropropagation*. Revi. Ed. Timber Press, Portland, OR.

Laimer, M., and Rèucker, W. (2003). *Plant tissue culture: 100 years since Gottlieb Haberlandt*. Springer Publishing Co., New York.

Lumsden, P. J., Nicholas, J. R., and Davies, W. J. (1994). *Physiology, growth, and development of plants in culture.* Kluwer Academic Publishers, Boston, MA.

Pierik, R. L. M. (1997). *In vitro culture of higher plants.* Kluwer Academic Publishers. Boston, MA.

Razdan, M. K. (2003). *Introduction to plant tissue culture.* 2nd ed. Science Publishers, Enfield, NH.

Reinert, J., and Bajaj, Y. P. S. (1977). *Applied and fundamental aspects of plant cell, tissue, and organ culture*. Springer-Verlag. New York.

Trigiano, R. N., and Gray, D. J. (2000). *Plant tissue culture concepts and laboratory exercises.* 2nd ed. CRC Press, Boca Raton, FL.